

## Preliminary Results

### Response of primary human epithelial cells to stringent virus amplification protocols

#### Summary

Exosomes are small extracellular vesicles containing cargo RNA, DNA and cellular proteins. They are produced by all cell types, add an additional layer to cell-to-cell communication and represent promising therapeutic targets. To investigate RNA species and extracellular vesicles produced under stringent protocols applied in virology, healthy primary human epithelial cells were cultured over three passages with routine starvation protocols for virus (virion) amplification. Despite lack of virus inoculation, cells exhibited severe cytopathic effects (CPE) resulting in visible subtotal destruction and plaque formation in continuous monolayer cultures. Blind inspection of cells under control and virus amplification conditions allowed to identify the different morphologies with a 100% hit rate. Total RNA combined from cells and cell culture supernatants of three biological and two technical replicates per starvation group was submitted for bulk next generation RNA sequencing along with total RNA from the same cells cultured under optimal conditions. Sequence and extracellular vesicle analyses are ongoing.

#### Introduction

Virus isolates, for example from bats, are amplified in cultured cells under conditions of starvation including the reduction of fetal calf serum (FCS) from 10% to 2% or 1% in Dulbecco's Modified Eagle's Medium (DMEM), also recommended by [ATCC](#). Starvation is further routinely combined with high concentrations of triple antibiotics from Gibco (penicillin/streptomycin antibiotics with amphotericin B antimycotic) and consecutive rounds of "blind" passaging of cell culture supernatants to fresh cell cultures.<sup>1</sup> Morphologically, virion amplification is expected to result in CPE characterized by cell rounding, ballooning and cellular degeneration, ultimately visible by plaque formation in a continuous cellular monolayer. Furthermore, viral particles, purified from these cell culture supernatants, have been illustrated by electron microscopy. To exclude the hypothesis that harsh starvation conditions in absence of virus inoculation can potentially lead to exosome formation<sup>2</sup> resembling virions, we subjected healthy primary human epithelial cells to routine virus amplification protocols. We then isolated total RNA from starved or control cells and supernatants with viral RNA isolation kits or routine TRIzol extraction and subjected the RNA to bulk next generation RNA sequencing.

#### Results

Healthy, primary human epithelial cells were cultured over four passages (P3-P6) under optimal culture conditions in defined epithelium control medium with 1x triple antibiotics. At each passage, the cell stock was divided in four groups. After 3 days in CM, the cultures were transferred to either fresh CM (CM, Control group 1), DMEM/GlutaMAX containing 10% FCS, 1x triple antibiotics (Control group 2) or starvation medium (Starvation group 1 & 2). During the first passage the starvation medium contained DMEM, 1% FCS and 3x triple antibiotics. The second and third passage were "blind" passages of 50% of culture supernatant carried forward from the last passage to the next one. The second starved group was additionally stressed at every passage with total yeast RNA (Yeast totalRNA, shortened to yRNA) for one hour prior to addition of the starvation medium (Starvation group 2).

Once transferred to DMEM with 10% FCS, epithelial cells adopted a flatter morphology than in CM and formed continuous cell sheets, due to high concentrations of calcium in DMEM, but otherwise continued to expand normally (Figure 1A). In contrast, cells in the starvation media contracted to small islands with reduced growth and started to degenerate. Over the next two passages, the cells incubated with supernatant from starved cells exhibited increasing CPE with cell free areas reminiscent of plaques in the cell sheet, and more dead cells in the supernatant (Figure 1B). Confluent cultures under starvation (Figure 1C) stained with crystal violet (Figure 1D) exhibited prominent CPE. Pyknotic cells with condensed nuclei and ballooning cells were predominantly present in Starvation group 1 and areas of total cellular destruction or plaques were present in Starvation group 1 but highest in number in Starvation group 2.

The experiments were conducted in three biological replicates in technical duplicates. All cultures were inspected blindly and the Starvation groups were easily recognized by drastic changes in morphology. At the end of three passages, RNA was collected from the Control 1 and both Starvation cell groups and supernatants, isolated with viral RNA kits or TRIzol and subjected to bulk next generation RNA sequencing. Isolated total RNA amounts were highest in the Control group 1 (Table 1) and had impeccable quality in all groups (data not shown). At end point, supernatants were harvested to investigate the presence of extracellular particles. Results are being processed.

## Material and Methods

### Cell culture

Commercial human primary epithelial cells were thawed at passage 3 and seeded at 4000 cells/cm<sup>2</sup> in 75cm<sup>2</sup> flasks for expansion at 37°C with 5% CO<sub>2</sub> in defined epithelial low calcium medium (without FCS) and 1x triple antibiotic solution (Gibco) (control medium, CM). At >80% confluence, the expansion cells were detached using 5mL accutase enzyme at 37°C for 10 minutes. Accutase was neutralized with 10mL CM, cells centrifuged for 5 minutes at 400G, resuspended in 1mL CM and counted with trypan blue staining using the Countess II instrument (ThermoFisher) to quantify live cells entering the experiments or parallel amplification rounds for subsequent passages.

For each experiment, four groups of healthy primary epithelial cells from the same expanded healthy stock were seeded in CM at 4000 cells/cm<sup>2</sup> in 25cm<sup>2</sup> flasks and cultured up to >50% confluence. The medium was then replaced by four experimental conditions; in control cells by fresh CM (Control group 1) or commercial DMEM supplemented with GlutaMAX, 10% heat-inactivated FCS and 1x triple antibiotic (Control group 2). Starvation was induced by replacing CM with DMEM supplemented with 1% FCS and 3x triple antibiotics following essentially the virion amplification protocols<sup>1</sup> (Starvation group 1 & 2). Starvation group 2 was additionally stressed with 10 µg total yeast RNA per flask for 1h and thoroughly washed with phosphate-buffered saline (PBS) before the medium change. Two blind passages carrying over 50% of the supernatant of Starvation groups 1 & 2 to the next corresponding cell culture were then performed alongside control groups which obtained 100% fresh medium. Supernatants were harvested, cleared from dead cells by centrifugation at 400G for 5 minutes and transferred to the next corresponding culture.

Experiments were repeated three times in duplicates. Starvation periods defined in the first biological replicate were held constant for all experiments. No medium change was performed during the starvation period.

P4: all conditions medium change around 50% confluence after seeding; control cells cultured until >80% confluence, starved cells cultured for 5 days after medium change.

P5: all conditions medium change around 50% confluence after seeding; control cells cultured until >80% confluence, stressed cells cultured for 8 days after medium change.

P6/RNA Isolation: all conditions medium change around 50% confluence after seeding; control cells cultured until >80% confluence, stressed cells cultured for 5 days after medium change.

P6/Crystal violet: all conditions medium change at 100% confluence after seeding; culture and stress induction for 3 days.

One representative micrograph of each flask was taken daily at room temperature using a Bright Field Nikon Eclipse TS100 microscope with a Nikon 1J5 camera, a Nikon FT1 adapter, and a 4x objective.

### **RNA extraction from epithelial cell cultures and supernatants**

At the endpoint of passage 6, half of the total cellular RNA was isolated with the Promega miRNA kit (Promega, Z6211), recommended for small and large RNA sampling, according to the manufacturer protocol. The other half of total cellular RNA was extracted with the standard TRIzol protocol. Total RNA from cell culture supernatant was isolated with the routinely used Qiagen viral RNA kit (Qiagen, 52904) according to the manufacturer protocol. All RNA samples were DNase treated. Total RNA concentration, as well as 260/280 and 260/230 ratios, were assessed using a NanoDrop 2000 (ThermoFisher). RNA amounts were highest in cells cultured in CM and lowest in Starvation groups 1 & 2 while supernatants had overall low but similar RNA levels (Table 1). 8.3 mg total RNA of high quality as assessed by Bioanalyzer from Control group 1 and Starvation groups 1 & 2 were subjected to bulk next generation RNA sequencing.

### **Crystal violet staining**

At the last passage, a second set of 25cm<sup>2</sup> flasks was seeded at 8000 cells/cm<sup>2</sup> (set 2) to assess cytopathic effects. At 100% confluence these cells were separately exposed to one of the four culture conditions. Within 3 days of exposure the cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature and then stained with 1% Crystal Violet for another 30 minutes at room temperature before thorough washing with tap water at room temperature.

The Bright Field Nikon Eclipse TS100 microscope with a Nikon 1J5 camera, a Nikon FT1 adapter, and a 4x or 20x objective were used to picture stained cultures.

### **References**

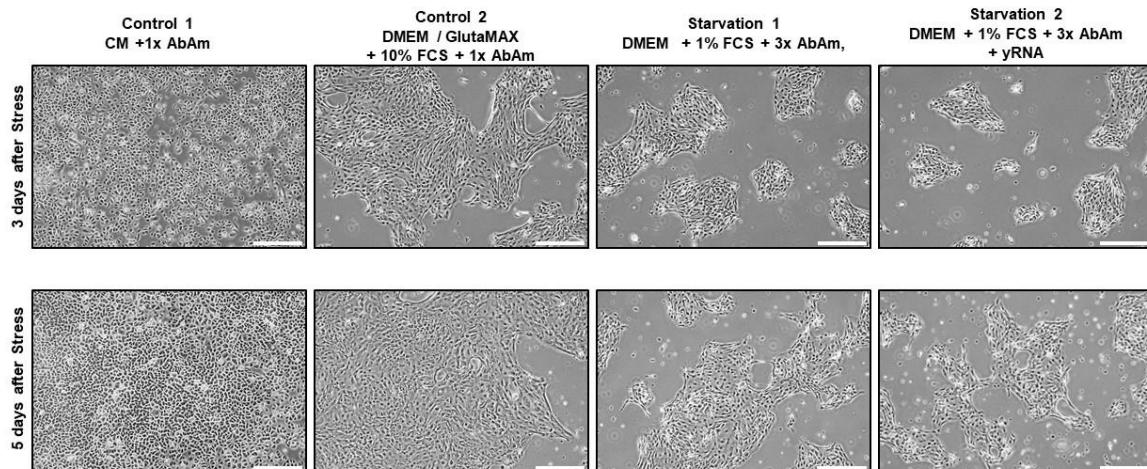
- 1 Ge, X. Y. et al. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature* **503**, 535-538, doi:10.1038/nature12711 (2013).

- 2 Gurung, S., Perocheau, D., Touramanidou, L. & Baruteau, J. The exosome journey: from biogenesis to uptake and intracellular signalling. *Cell Commun Signal* **19**, 47, doi:10.1186/s12964-021-00730-1 (2021).

## Figures

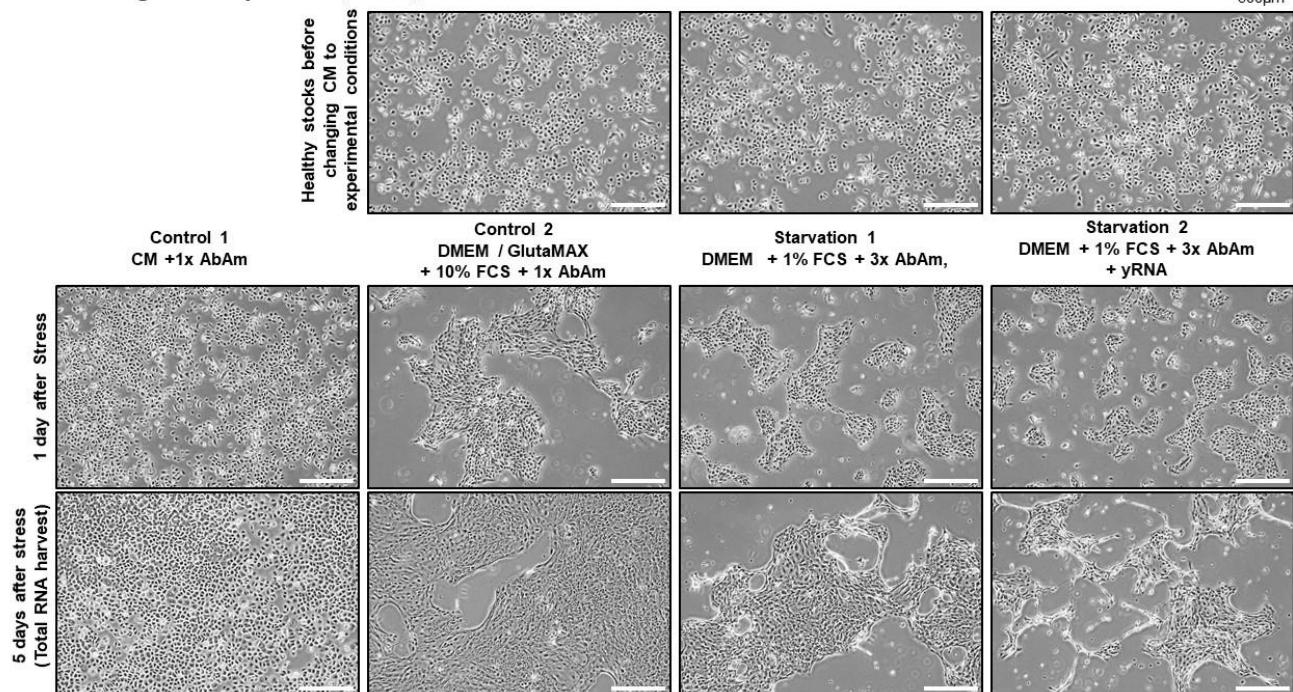
500μm

### A. Passage 4 – Expansion

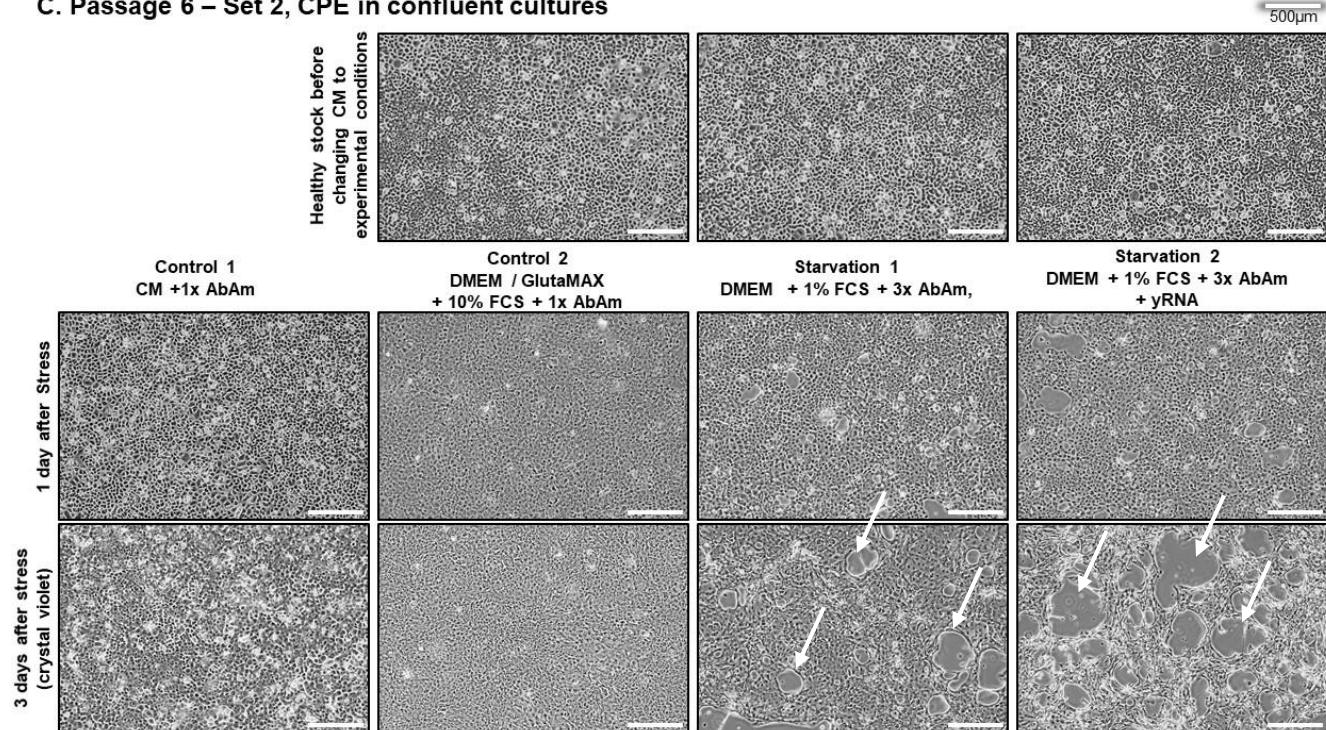


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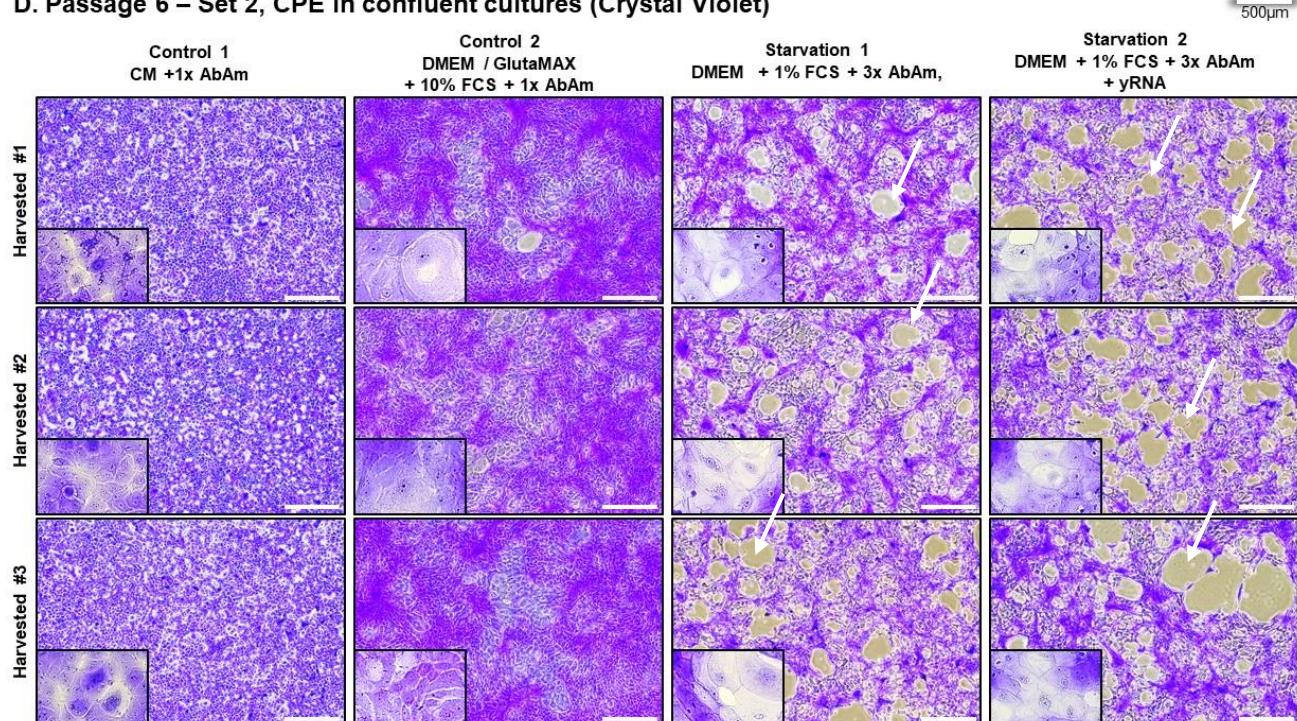
### B. Passage 6 – Expansion, Set 1, RNA harvest



**C. Passage 6 – Set 2, CPE in confluent cultures**



**D. Passage 6 – Set 2, CPE in confluent cultures (Crystal Violet)**



**Figure 1. Starvation of epithelial cells.** Representative micrographs of the 4 experimental groups of epithelial cells at passage 4 and 6 as indicated. From left to right: healthy control cells with 1x triple antibiotics in control medium (CM) or DMEM/GlutaMAX with 10% FCS, starved cells with 3x triple antibiotics and DMEM with 1% FCS. Right panel cells were treated with total yeast RNA (yRNA) for 1h prior to medium switch. (A), (B) Cells under expansion over time serving RNA isolation; note that CPE become more prominent over the three passages. (B) Top raw: cells before medium switch. (C), (D) Confluent cells to visualize CPE;

(C) Top raw: confluent cells before medium switch. (D) Cell cultures from 3 biological replicates stained with crystal violet at the time of harvest. Note that cells in the left two panels form a continuous cell sheet while cells in the right two panels exhibit a high number of plaques (arrows) compatible with significant cytopathic effects increasing from day 1 to day 5. Yeast RNA treated cultures show a significantly higher number and larger plaques. Inserts: 20x magnification; some rare pyknotic and ballooning cells were observed in control cultures; ballooning cells with void cytoplasm are most frequent under Stress conditions 1. Cultures were inspected blindly by 2 experimenters once a day with a 100% hit rate. Scale bars; 500 µm. All cultures: n=3 in duplicates.

**Table 1: RNA isolation scheme**

Groups #	Label	Sample Percentile	Harvesting Method	Total RNA in µg	RNA Vol. in µL
Control 1 CM + 1x AbAm	Control 1	100% Supernatant	Viral RNA Kit (Column)	<b>0,27</b>	30,00
		100% Cells	miRNA Kit+TRIzol	<b>21,53</b>	15,00
Control 2 DMEM + GlutaMAX + 10% FCS + 1x AbAm	Control 2	100% Supernatant	Viral RNA Kit (Column)	<b>0,26</b>	30,00
		100% Cells	miRNA Kit+TRIzol	<b>14,78</b>	15,00
Stress 1 DMEM + 1% FCS + 3x AbAm	Starvation 1	100% Supernatant	Viral RNA Kit (Column)	<b>0,32</b>	30,00
		100% Cells	mRNA Kit+TRIzol	<b>8,32</b>	15,00
Stress 2 DMEM + 1% FCS + 3x AbAm + Yeast tRNA	Starvation 2	100% Supernatant	Viral RNA Kit (Column)	<b>0,27</b>	30,00
		100% Cells	miRNA Kit+TRIzol	<b>9,25</b>	15,00